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PCR Detection of Metallo-β-Lactamase Gene (bla_{IMP}) in Gram-Negative Rods Resistant to Broad-Spectrum β-Lactams

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We applied PCR to the rapid detection of the metallo-β-lactamase gene, bla_{IMP}, in clinically isolated gram-negative rods. A total of 54 high-level ceftazidime-resistant strains (MICs, >128 μg/ml) were subjected to PCR analyses with the bla_{IMP}-specific primers, since the bla_{IMP}-bearing clinical isolates tested in our previous study always demonstrated high-level resistance to ceftazidime. Twenty-two bla_{IMP}-positive strains including 9 *Pseudomonas aeruginosa*, 9 *Serratia marcescens*, 2 *Alcaligenes xylosoxidans*, 1 *Pseudomonas putida*, and 1 *Klebsiella pneumoniae* strains were newly identified from 18 different hospitals in Japan. These strains were mostly isolated from urine samples and showed high-level resistance to almost every cephem, while their levels of resistance to carbapenems were diverse. The PCR analyses with novel integrase gene-specific (*intI3*) and *acc(6′)-Ib* gene-specific primers suggested that the integron structure found in a large plasmid harbored by *S. marcescens* AK9373 was also well conserved among bla_{IMP}-positive strains. These results imply that the bla_{IMP} gene cassettes have been dispersing into various gram-negative rods with the help of the newly identified integron element. Thus, the PCR-aided rapid detection will be helpful for the early recognition of emerging bla_{IMP}-positive clinical isolates which demonstrate consistent resistance to β-lactams.

Among the various β-lactams, carbapenems are potent agents for the chemotherapy of infectious diseases caused by gram-negative rods, since they are quite stable against the β-lactamases produced by these organisms (5). However, recently, the numbers of carbapenem-resistant clinical isolates which belong to the family *Enterobacteriaceae* or pseudomonads have been increasing in Japan (4, 7, 8, 19), and some of them were found to produce a new metallo-β-lactamase, IMP-1, which efficiently hydrolyzes carbapenems as well as broad-spectrum β-lactams (3, 12, 15, 16). Moreover, IMP-1 is notable for its special character, in that it is hardly blocked by β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam (11). Therefore, strains producing IMP-1 are difficult to control with β-lactams and related drugs in combination. Furthermore, we have reported that the metallo-β-lactamase gene (bla_{IMP}) cassette of *Serratia marcescens* AK9373 is located in the space between the integrase gene (*intI3*) and the aminoglycoside acetyltransferase gene [*aac(6′)-Ib*] (1) and is transposed into other plasmids or the chromosomes of gram-negative bacteria by this integron element, which is mediated by large plasmids with wide host ranges. Int13 has been newly identified as the third class of integrase (14), showing 60.9% amino acid sequence similarity to the reported integrase (IntI1) (2). This finding warned of the further dissemination of the bla_{IMP} gene cassette into various gram-negative rods. Quite recently, we also reported multifocal outbreaks of IMP-1-producing *Pseudomonas aeruginosa* isolates in Japan (17). Hence, we have been directing special attention to the early recognition of metallo-β-lactamase-producing clinical isolates. According to our previous studies of bla_{IMP}-positive strains, the results of susceptibility tests were not enough for the identification of bla_{IMP}-positive strains, because several clinical isolates carrying a cryptic bla_{IMP} gene demonstrated low-level carbapenem resistance (MICs, ≤4 μg/ml). However, the level of resistance to carbapenems in these strains might be elevated through enhanced expression of the bla_{IMP} promoter in the presence of broad-spectrum cephems or carbapenems. Hence, early recognition of bla_{IMP}-positive strains would be essential for the selection of appropriate antimicrobial agents for chemotherapy. In this study, therefore, ceftazidime-resistant gram-negative rods isolated from different hospitals in Japan were subjected to a PCR-aided rapid detection method with the bla_{IMP}-specific primers.

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MATERIALS AND METHODS

Bacterial strains. A total of 54 clinical isolates of gram-negative rods which demonstrated high-level resistance to ceftazidime (MICs, >128 μg/ml) were subjected to PCR for detection of the bla_{IMP} gene, since in our previous study all *S. marcescens* and *P. aeruginosa* strains that carry the bla_{IMP} gene belonged to this group (3, 12, 17). Thirty-three strains of *P. aeruginosa* and 1 strain of *S. marcescens* were provided by the Working Group on Antimicrobial Resistance Survey in Japan in 1994. Nine *S. marcescens* strains, five *P. aeruginosa* strains, and 1 *Klebsiella pneumoniae* strain were provided by Health Science Research Institute in 1995. Two *P. aeruginosa* strains were isolated at the Kyushu University Hospital in 1995, and two *Alcaligenes xylosoxidans* strains were isolated at the Nagoya University Hospital in 1994. One *Pseudomonas putida* strain was isolated at the Saga Medical School Hospital in 1994. All the bla_{IMP} gene-positive strains, including 15 previously reported *P. aeruginosa* strains (17) and 5 *S. marcescens* strains (3, 12), were subjected to PCR analyses to detect the *intI3* and the *aac(6′)-Ib* genes.

Antimicrobial agents and susceptibility tests. The following antibiotics used in this study were provided by the indicated sources: amikacin, Bristol-Myers Squibb K.K., Tokyo, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; biapenem, Lederle (Japan), Ltd., Tokyo, Japan; BO2727 (9) (a new carbapenem) and imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; cefpirome, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan; cefoperazone-sulbactam, Pfizer Pharmaceutical Inc., Tokyo, Japan; cefsulodin, Takeda Chemical Industries, Ltd., Osaka, Japan; ceftazidime, Japan Glaxo Co., Tokyo, Japan; ciprofloxacin, Bayer Yakuhin, Ltd., Osaka, Japan; meropenem, Sumitomo Pharmaceutical Ltd., Osaka, Japan; moxalactam, Shionogi & Co., Ltd., Osaka, Japan; panipenem, Sankyo Co., Ltd., Tokyo, Japan; and piperacillin, Toyama Chemical Co., Ltd., Toyama, Japan.

Antimicrobial susceptibility testing was performed by the agar dilution method according to National Committee for Clinical Laboratory Standards document

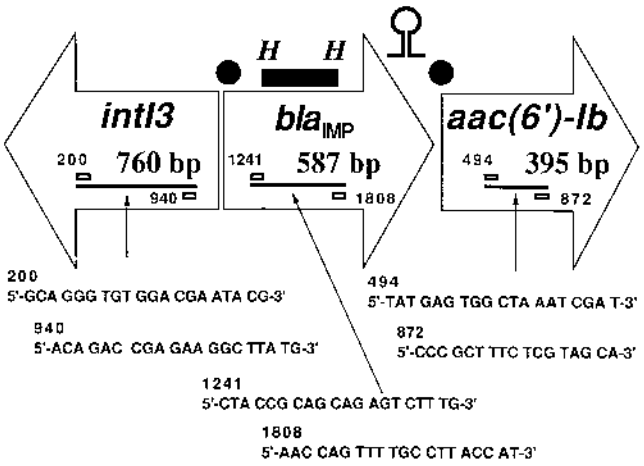

FIG. 1. Specific primer design for bla_{IMP}, *intI3*, and *aac(6′)-Ib* genes. The sequence and position of each primer are shown. The open arrows indicates the positions and transcriptional directions of the bla_{IMP}, *intI3*, and *aac(6′)-Ib* genes found in *S. marcescens* AK9373 (1). The thick horizontal line indicates the position of the bla_{IMP}-specific hybridization probe used in our previous study (3, 17). The positions of the GTTRRRY-like sequences (●) and an atypical 59-base element containing a 67-bp inverted repeated sequence (Ω), which were speculated to be involved in the integrase-dependent recombination in *S. marcescens* AK9373, are shown above the bla_{IMP} gene. The positions of sense and antisense primers for *aac(6′)-Ib* refer to the nucleotide sequence published by Ploy et al. (13).

M7-A3 (10). Mueller-Hinton II agar and Mueller-Hinton II broth (BBL Microbiology Systems, Cockeysville, Md.) were used for susceptibility testing.

Design of oligonucleotide primers. The sequences and locations of the primers used in the PCR are listed in Fig. 1. The bla_{IMP} gene- and *intI3* gene-specific primers were designed with OLIGO software, version 4.0 (Takara Shuzo Co., Ltd., Shiga, Japan). For the sequence of *aac(6′)-Ib* gene-specific primers, we referred to previously published data (13).

PCR procedure. Template DNAs were prepared from each strain as described previously (18). PCR amplification was performed in a 50-μl volume with the GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, Conn.) thermal cycler. Reaction mixtures contained 1 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate (Takara Shuzo Co., Ltd.), 1× reaction buffer containing 1.5 mM $MgCl_2$ (Takara Shuzo Co., Ltd.), 2.5 U of TaKaRa *Taq* polymerase (Takara Shuzo Co., Ltd.), and approximately 25 ng of template DNA. After an initial denaturation step (2 min at 94°C), 30 cycles of amplification were performed, as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and DNA extension at 72°C for 1.5 min. After agarose gel electrophoresis, the ethidium bromide-stained PCR products were visualized under UV light. In the amplification of the *intI3* gene, the annealing step was carried out at 57°C. For more rapid and convenient template DNA extraction, the supernatant of a bacterial culture suspension boiled for 10 min was applied as described previously (6).

RESULTS AND DISCUSSION

For the preliminary study, 5 *S. marcescens* isolates (3, 12) and 15 *P. aeruginosa* isolates (17) which had been confirmed to carry the bla_{IMP} gene by DNA hybridization analyses in our previous study were subjected to PCR analysis, and the bla_{IMP}-specific fragments in all these isolates were amplified. Examples of the results of PCR analysis are presented in Fig. 2. In contrast, no bla_{IMP}-specific fragment was amplified in hybridization-negative *S. marcescens* and *P. aeruginosa* strains under the same experimental conditions. Thus, this PCR method was helpful for the rapid detection of the bla_{IMP} gene. Then, 54 high-level ceftazidime-resistant clinical isolates (MICs, >128 μg/ml) were screened by this method, and 9 *P. aeruginosa* isolates and 9 *S. marcescens* isolates were newly found to carry the bla_{IMP} gene. The bla_{IMP} gene was also detected in two *A. xylosoxidans* isolates, one *P. putida* isolate, and one *K. pneumoniae* isolate. The locations of the hospitals where bla_{IMP}-positive strains were isolated are indicated in Fig. 3. Indeed, the plasmid-mediated bla_{IMP} gene was initially found in *S. marcescens* and *P. aeruginosa* isolates, but this is the first report of *A. xylosoxidans* and *P. putida* clinical isolates that carry the bla_{IMP} gene. The bla_{IMP} gene detected in these isolates might well be derived from *S. marcescens* or *P. aeruginosa* isolates by the help of transferable plasmids. The bla_{IMP} gene was detected in both *S. marcescens* and *K. pneumoniae* strains from a hospital in Kanagawa. From a hospital in Aichi, the bla_{IMP} gene was also detected in a *P. aeruginosa* strain and two *A. xylosoxidans* strains. Additionally, three bla_{IMP}-positive strains of *P. aeruginosa* and one bla_{IMP}-positive strain of *S. marcescens*

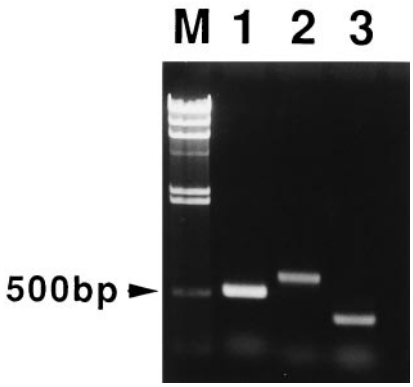

FIG. 2. PCR detection of bla_{IMP}, *intI3*, and *aac(6′)-Ib* in *S. marcescens* AK9373 (1). The sequences of the primers are presented in Fig. 1. Lanes: M, *Hin*dIII-digested DNA marker; 1, 587 bp PCR product of bla_{IMP} gene; 2, 760-bp PCR product of the *intI3* gene; 3, 395-bp PCR product of the *aac(6′)-Ib* gene.

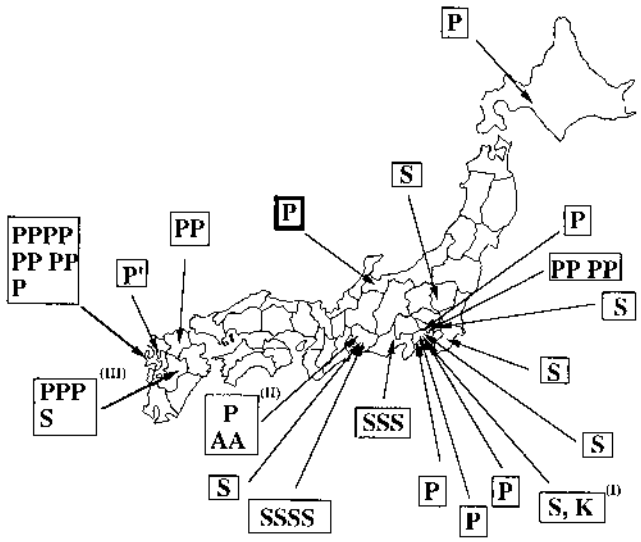

FIG. 3. Geographical distribution of hospitals where bla_{IMP} gene-carrying strains were isolated. The locations of each hospital where bla_{IMP}-carrying strains were isolated are indicated with a box. P, each bla_{IMP}-positive *P. aeruginosa* isolate; S, each bla_{IMP}-carrying *S. marcescens* isolate; A, each bla_{IMP}-carrying *A. xylosoxidans* isolate; P′, a bla_{IMP}-carrying *P. putida* isolate; K, a bla_{IMP}-positive *K. pneumoniae* strain; the P in the bold box indicates Toyama, where the first isolation of a transferable carbapenem-resistant *P. aeruginosa* was reported (19). (I), isolate from a hospital in Kanagawa; the bla_{IMP} gene was detected in both *S. marcescens* and *K. pneumoniae* strains; (II), isolate from a hospital in Aichi; the bla_{IMP} gene was also detected in a *P. aeruginosa* strain and two *A. xylosoxidans* strains; (III), three bla_{IMP}-positive strains of *P. aeruginosa* and one bla_{IMP}-positive strain of *S. marcescens* were identified from a hospital in Kumamoto.

TABLE 1. Susceptibilities of bla_{IMP}-carrying strains

Strain	MIC (μg/ml)[a]													
	IPM	PAPM	MEPM	BIPM	BO2727	PIPC	AZTR	CAZ	CPZ-SBT	CPR	LMOX	CFS	CPFX	AMK
P. aeruginosa														
MKAM12	8	64	64	8	64	>128	16	>128	>128	>128	>128	>128	128	16
MKBM18	32	>128	>128	64	>128	32	8	>128	>128	>128	>128	>128	128	>128
MKCM43	128	>128	128	64	8	>128	>128	128	>128	>128	>128	>128	8	32
MTA811	4	32	16	16	16	8	8	>128	>128	>128	>128	>128	<0.5	32
MTA852	8	32	16	8	8	16	16	>128	>128	128	>128	>128	<0.5	32
MNG1059	16	64	>128	64	>128	64	64	>128	>128	128	>128	>128	2	16
MNA1441	32	>128	>128	>128	>128	128	64	128	>128	32	>128	>128	16	16
MKK87B	32	>128	128	64	64	64	32	>128	>128	>128	>128	>128	32	16
MKK119A	64	>128	>128	>128	128	32	16	>128	>128	>128	>128	>128	128	16
S. marcescens														
MKU15140	128	>128	128	64	128	>128	8	128	>128	32	>128	>128	16	64
MTIM2	>128	>128	>128	>128	>128	>128	8	>128	>128	>128	>128	>128	>128	>128
MKDM17	>128	>128	>128	>128	>128	>128	8	>128	>128	>128	>128	>128	16	128
MTCM35	>128	>128	>128	>128	>128	>128	8	>128	>128	>128	>128	>128	16	64
MSIM36	>128	>128	>128	>128	>128	>128	8	>128	>128	128	>128	>128	8	16
MSIM44	>128	>128	>128	>128	>128	>128	8	>128	>128	128	>128	>128	8	16
MSIM60	>128	>128	>128	>128	>128	>128	16	>128	>128	>128	>128	>128	4	16
MKEM56	>128	>128	128	128	128	>128	4	>128	>128	>128	>128	>128	8	64
MTOM58	>128	>128	>128	>128	>128	64	8	>128	>128	>128	>128	>128	64	64
A. xylosoxidans														
MNG10131	8	32	8	32	>128	1	64	128	128	>128	>128	>128	16	128
MNG1060	8	32	8	32	>128	1	64	128	128	>128	>128	>128	16	>128
P. putida MSGD1	128	>128	>128	>128	>128	32	>128	>128	>128	128	>128	>128	16	4
K. pneumoniae MKD115	64	128	64	32	128	>128	0.5	>128	>128	>128	>128	>128	<0.5	2

[a] Abbreviations: IPM, imipenem; PAPM, panipenem; MEPM, meropenem; BIPM, biapenem; PIPC, piperacillin; AZTR, aztreonam; CAZ, ceftazidime; CPZ-SBT, cefoperazone-sulbactam; CPR, cefpirome; LMOX, moxalactam; CFS, cefsulodin; CPFX, ciprofloxacin; AMK, amikacin.

were identified from a hospital in Kumamoto. In each hospital, plasmid-mediated intergenic transfer of the bla_{IMP} gene was strongly suspected. Hence, we must face the facts that the bla_{IMP} gene-bearing element has been dispersing among gram-negative organisms.

The newly identified 22 bla_{IMP}-positive strains showed consistent high-level resistance to almost all broad-spectrum cephems including ceftazidime and cefoperazone-sulbactam (MICs, ≥128 μg/ml), but their levels of resistance to carbapenems were diverse (Table 1). Expression of the bla_{IMP} gene might be cryptic or suppressed in the strains demonstrating low-level carbapenem resistance. In our previous work, cryptic bla_{IMP} genes were also identified in several isolates of *S. marcescens* (3) and *P. aeruginosa* (17). After long-term exposure to carbapenems, however, high-level carbapenem-resistant strains may emerge from the cryptic strains through elevated expression of the bla_{IMP} gene because of mutations in its promoter region. Since antimicrobial susceptibility testing does not suffice for the early identification of the bla_{IMP}-carrying strains, it is worthwhile to subject every clinical isolate demonstrating high-level resistance to ceftazidime and/or cefoperazone-sulbactam (MICs, ≥128 μg/ml) to this PCR method for the detection of the bla_{IMP} gene.

Although all the bla_{IMP}-positive strains tested in this study demonstrated similar susceptibilities to five carbapenems, their susceptibilities to piperacillin and aztreonam did not parallel those to carbapenems. Aztreonam is notable for its relatively low MIC for IMP-1-producing isolates of *S. marcescens*. These findings may suggest the presence of alternative resistance mechanisms in high-level aztreonam-resistant strains such as MKCM43. Several bla_{IMP}-positive strains tested in this study were still susceptible to ciprofloxacin and amikacin. Therefore, susceptibilities to new quinolones and aminoglycosides should be tested for clinical isolates which are suspected of carrying the bla_{IMP} gene.

The sources of the bla_{IMP}-positive strains were as follows. Six *P. aeruginosa* strains, eight *S. marcescens* strains, one *P. putida* strain, and one *K. pneumoniae* strain were isolated from urine samples. Two strains of *A. xylosoxidans* were from pharyngeal exudates. One *P. aeruginosa* strain was from bile. One strain of *S. marcescens* was from sputum. Broad-spectrum β-lactams are usually administered to inpatients through drip infusion, and they are mostly excreted in urine or bile without being dissolved or modified. Since these agents tend to be highly condensed in urine, bacteria surviving in the urinary tract must acquire high-level resistance to these agents. For this purpose, metallo-β-lactamase production is of great benefit to these bacteria. Therefore, it is quite natural that the bla_{IMP}-positive strains were often isolated from urine samples. When broad-spectrum cephems are administered, repeat identification and susceptibility testing of bacteria from urine samples, as well as the PCR analyses, would be helpful for the early recognition of bla_{IMP}-positive strains. Moreover, a method for the direct detection of the bla_{IMP} gene from the urine samples of patients infected with high-level cephem-resistant strains remains to be established.

Results of PCR detection of the *intI3* gene and the *aac(6′)-Ib* gene are listed in Table 2. The *intI3* gene was detected in 19 of the 24 bla_{IMP}-positive *P. aeruginosa* strains, 11 of the 14 bla_{IMP}-positive *S. marcescens* strains, 1 of 2 bla_{IMP}-

TABLE 2. PCR detection of *intI3* and *aac(6′)-Ib* in bla_{IMP}-positive strains

Strain	*intI3*	*aac(6′)-Ib*
P. aeruginosa		
MNA1428	−	+
MNA1455	+	+
MNA14102	+	+
MNA14115	+	+
MNA14141	−	+
MNA14109	+	+
MNA1443	+	+
MNA1417	+	+
MKU1560	+	+
MKU1550	+	+
MKU1563	−	−
MTA815	+	+
MTA854	+	+
MTB9131	+	+
MSA137	+	−
MKAM12	+	+
MKBM18	−	+
MKCM43	+	+
MTA811	+	+
MTA852	+	+
MNG1059	+	+
MNA1441	+	+
MKK87B	−	+
MKK119A	+	−
S. marcescens		
TN9106	+	−
AK9373	+	+
AK9374	+	+
AK9385	+	+
AK9391	+	+
MKU15140	+	+
MTIM2	+	+
MKDM17	+	−
MTCM35	+	−
MSIM36	−	+
MSIM44	−	+
MSIM60	−	+
MKEM56	+	−
MTOM58	+	−
A. xylosoxidans		
MNG1060	+	−
MNG10131	−	−
P. putida MSGD1	+	−
K. pneumoniae MKD115	+	+

positive *A. xylosoxidans* strains, 1 bla_{IMP}-positive *P. putida* strain and 1 of bla_{IMP}-positive *K. pneumoniae* strain. The *aac(6′)-Ib* gene was also amplified from 21 of the 24 bla_{IMP}-positive *P. aeruginosa* strains, 9 of the 14 bla_{IMP}-positive *S. marcescens* strains, and 1 bla_{IMP}-positive *K. pneumoniae* strain. These results imply that the *intI3* and *aac(6′)-Ib* genes are well conserved among these bla_{IMP}-positive strains. Hence, these findings also suggest that the bla_{IMP} gene cassettes have already been widely disseminated and will further disperse into various plasmids or chromosomes of gram-negative rods with the newly identified integron element.

In the present study, two different methods for the preparation of the DNA template were used. When the supernatant of a bacterial culture suspension boiled for 10 min was applied as a DNA template, we were unable to amplify the bla_{IMP} gene in 1 of 24 bla_{IMP}-positive *P. aeruginosa* strains and 4 of 14 *S. marcescens* strains. This might be due to the production of DNase or an inhibitor of *Taq* DNA polymerase, because we succeeded in amplifying the bla_{IMP} gene when DNA templates prepared by a conventional method (18) were used. For the rapid and accurate detection of the bla_{IMP} gene in clinical laboratories, more convenient methods for the preparation of DNA templates should be established.

In ordinary cases, it takes at least 3 days to receive the results of susceptibility tests from clinical laboratories, which might be too late to select appropriate antimicrobial agents for therapy. Therefore, the PCR-aided rapid detection method described here would provide a clue for rigorous infectious disease control. Moreover, the PCR detection method will contribute to the prevention of interhospital transfer of these bla_{IMP}-carrying microorganisms.

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